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Gr and hp-1 tomato mutants unveil unprecedented interactions between arbuscular mycorrhizal symbiosis and fruit ripening

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Abstract

Main conclusion Systemic responses to an arbuscular mycorrhizal fungus reveal opposite phenological patterns in two tomato ripening mutants depending whether ethylene or light reception is involved. The availability of tomato ripening mutants has revealed many aspects of the genetics behind fleshy fruit ripening, plant hormones and light signal reception. Since previous analyses revealed that arbuscular mycorrhizal symbiosis influences tomato berry ripening, we wanted to test the hypothesis that an interplay might occur between root symbiosis and fruit ripening. With this aim, we screened seven tomato mutants affected in the ripening process for their responsiveness to the arbuscular mycorrhizal fungus Funneliformis mosseae. Following their phenological responses we selected two mutants for a deeper analysis: Green ripe (Gr), deficient in fruit ethylene perception and high-pigment-1 (hp-1), displaying enhanced light signal perception throughout the plant. We investigated the putative interactions between ripening processes, mycorrhizal establishment and systemic effects using biochemical and gene expression tools. Our experiments showed that both mutants, notwithstanding a normal mycorrhizal phenotype at root level, exhibit altered arbuscule functionality. Furthermore, in contrast to wild type, mycorrhization did not lead to a higher phosphate concentration in berries of both mutants. These results suggest that the mutations considered interfere with arbuscular mycorrhiza inducing systemic changes in plant phenology and fruits metabolism. We hypothesize a cross talk mechanism between AM and ripening processes that involves genes related to ethylene and light signaling.

Keywords Arbuscular mycorrhiza - Ethylene – Light signaling - Ripening mutants - Tomato - *Funneliformis mosseae*

Abbreviations

AM Arbuscular mycorrhiza

AMF Arbuscular mycorrhizal fungi

ET Ethylene

ETRs Ethyelene receptors

MYC Mycorrhizal

NM Non-mycorrhizal

Introduction

Arbuscular mycorrhiza (AM) is a mutualistic symbiotic association that involves arbuscular mycorrhizal fungi (AMF), belonging to the Glomeromycota, and 80 % of land plant species, including many agricultural crops. Both partners gain an advantage from the symbiosis; the fungus supplies the plant with mineral nutrients (mainly phosphate) increasing, plant biomass and improving its resistance to pathogens and environmental stresses including oxidative stress conditions (Garcia-Sànchez et al. 2014), while the host provides organic carbon to the fungus (Bonfante and Genre 2010). Plant and fungal nutrition is thus mutually improved (Kiers et al. 2011). Experimental evidence reveals that AMF both improve plant health and growth as natural biofertilisers and enhance organoleptic and nutritional traits of edible crops (Salvioli and Bonfante 2013). Among them, tomato, one of the most widely grown crops worldwide, displays a relevant responsiveness to AMF (Regvar et al. 2003; Copetta et al. 2011). Tomato represents an important component of human diets, providing a major source of provitamin A and vitamin C, in addition to antioxidant carotenoids. New platforms, including high throughput sequencing, have recently revealed the wide molecular reprogramming that occurs during mycorrhizal symbiosis and allowed the description of a set of mycorrhiza-induced root-specific genes. These genes regulate not only nutrient transport, but also carbohydrate and lipid metabolism as well as cell wall remodeling (Ruzicka et al. 2013). Extending transcriptomic analysis to the whole plant, an organ-independent gene core set and a systemic defence-related gene program have been detected in shoot and fruits, both induced by the presence of AMF (Fiorilli et al. 2009; Salvioli et al. 2012). Fruits from mycorrhizal (MYC) tomato plants have also been found to be enriched in healthy compounds, including amino acids (Salvioli et al. 2012) and lycopene (Regvar et al. 2003). In addition, they possess a higher anti-oestrogenic power and no in vitro genotoxic effects (Giovannetti et al. 2012), as well as an enhanced expression of allergen-encoding genes that do not match with a higher allergenic potential for humans (Schwarz et al. 2011). A deeper mRNA-seq transcriptome analysis (Zouari et al. 2014) provided a more exhaustive molecular framework for fruits from tomato MYC plants. This study highlighted a fruit systemic ‘‘signature’’ consisting of climacteric fleshy fruit-specific genes as well as of transcripts commonly regulated by symbiosis. The differentially regulated genes refer principally to primary metabolic processes (e.g. photosynthesis, photorespiration, stress response, amino acid synthesis and transport, and carbohydrates metabolism), suggesting that AM symbiosis has deep impacts on fruit source-sink dynamics. Pathways associated with cell wall metabolism were down-regulated, including genes coding for polygalacturonase, which is directly involved in fruit ripening-associated cell wall remodeling. Since genes involved in ethylene (ET) metabolism were also repressed, Zouari et al. (2014) suggested that AM may also increase tomato shelf-life. The use of several tomato ripening mutants has shed light on the genetics behind fleshy fruit ripening mechanisms and their implications in plant hormone and light signal reception (Klee and Giovannoni 2011). Tomato ET mutants have been used to dissect the role of this hormone in AM establishment, since it has been suggested as a negative regulator of the symbiosis (Zso¨go¨n et al. 2008; Varma Penmetsa et al. 2008; Lo´pez-Ra´ez et al. 2010; Khatabi and Scha¨fer 2012). In this context, attention has been devoted to mutants affected in ET perception and synthesis (Barry and Giovannoni 2007) including epinastic (epi), ripening inhibitor (rin) or Never-ripe (Nr), and their mycorrhizal phenotypes have been described. Zso¨go¨n et al. (2008) used Nr (insensitive to ET) and epi (constitutive ET response phenotype) lines, and demonstrated a reduction in AMF colonization for both mutants. However, some conflicting data have been reported, as AMF colonization was positively affected using the same Nr mutant in another experiment (Fracetto et al. 2013). Lastly, the rin mutant, altered in multiple ripening phenomena including ET production, supported an enhanced AMF colonization level, suggesting that the RIN-pathway may regulate AM establishment via an ET-independent pathway (Torres de Los Santos et al. 2011). To contribute to this still largely

uncharacterized issue, i.e. the potential for cross talk between root AM symbiosis and fruit ripening in tomato, we screened seven mutants for their mycorrhizal phenotype at root level and their systemic responsiveness to AMF inoculation. Two out of them (rin and Nr) have already been tested by the previously reported groups, while the other four have never been screened for their mycorrhizal susceptibility. Mutants were grown in parallel to their isogenic line (Ailsa Craig) and inoculated with the AM fungus Funneliformis mosseae. As a further step, we selected two of them: Green ripe (Gr) which possesses a fruitspecific deficiency in ET perception (Barry and Giovannoni 2006), and high pigment 1 (hp-1) which presents a tomato UV-DAMAGED DNA-BINDING PROTEIN-1 (DDB1) gene mutation. This mutation results in constitutive light signal transduction that affects all plant organs and leads to elevated green fruit chlorophyll and ripe fruit carotenoid accumulation (Liu et al. 2004). These two mutants were investigated by applying molecular and biochemical approaches to compare the effect of AM symbiosis in roots versus fruits. The results indicated that mutations in ET and light perception do not have a clear cut impact on mycorrhizal phenotype, but have a different impact on mycorrhizal functioning and systemic mycorrhizal-induced traits, suggesting that some aspects of ripening, such as those regulated by light and ET signaling, are influenced by AM.

Materials and methods

Plant material and experimental design

Tomato (Solanum lycopersicum L.) ripening mutant (Table S1) seeds and their isogenic wild type genotype cv. Ailsa Craig (WT) were sterilized with a single wash (3 min) in 70 % ethanol with 3–4 drops of Tween®20

(Sigma-Aldrich) added, a step in 5 % commercial bleach solution (13 min) followed by three repeated 10 min washes in sterile water. Seeds were then germinated in 0.6 % plant agar medium and kept for 5 days in the dark at 23°C. After seed germination, petri dishes were kept in the light for 4 days [16 h light (23 °C)/8 h dark (21 °C)]. Two independent sets of plants were potted, selecting only healthy and fully germinated seedlings. For the assessment of mycorrhizal phenotype, one set of three WT and mutant seedlings (24 plants in all) were transferred in 10x10x12 cm pots and grown in a controlled growth chamber [14 h light (24°C)/10 h dark (20°C)] for 60 days. Mycorrhizal inoculation was achieved using a monospecific AMF inoculum of Funneliformis mosseae (syn. Glomus mosseae) purchased from MycAgro Lab. (Dijon, France), 30 % diluted in sterile quartz sand and pumice mixture. These substrates were separately sterilized in a drying oven at 180°C for 3 h. The top layer of AMFinoculated pots was filled with a small amount of quartz sand to avoid accidental spore aerial cross-contamination. The second experiment was set up to follow fruiting: five seedlings for both mycorrhizal (MYC) and non-mycorrhizal (NM) conditions for each genotype (a total of 80 plants) were potted after 10 days from the sowing in 14x14x16 cm pots as described above and grown in a greenhouse with natural light conditions from December 2010 to September 2011. For NM condition, a simple sand/pumice control growth substrate was used. For both the experimental sets, pots with newly planted seedlings were covered with nylon bags for 48 h to permit a gradual adaptation to the new light and moisture conditions, avoiding desiccation. Plants in all treatments were watered twice a week with tap water and once a week with a modified Long-Ashton nutrient solution containing a middle-strength phosphorus concentration (300 μM Na_2HPO_4) (Hewitt 1966).

Assessment of mycorrhizal colonization, phenological observations and sampling

Mycorrhizal establishment was assessed on plants 60 DPI (days post inoculation). The presence of extraradical mycelium (ERM) was firstly assessed using a stereomicroscope and then roots were stained for 12 h in 0.1 % w/v cotton blue in lactic acid and clarified in pure lactic acid. Roots were placed on glass slides and observed under optical microscope. Fungal colonization was then estimated according to Trouvelot et al. (1986). For each plant 100 pieces of 1 cm were analyzed, with three biological replicates per genotype. The colonization parameters were calculated using an Excel programmed sheet. Mycorrhizal colonization was similarly assessed on the greenhouse plant material at the end of the cultural cycle. To monitor the reproductive phenology of tomato plants growing in greenhouse, the number of days to flowering and the number of produced flowers and fruits were weekly recorded. For gene expression analysis, fruits were collected at 35 days after flowering at the mature green (MG) stage (Giovannoni 2004). At this stage, the fruit surface is completely green in color and photosynthesis reaches its maximum activity. Seeds and placental tissues were removed from the fruit and small pericarp pieces were immediately frozen in liquid nitrogen, freeze-dried overnight and stored at -80°C until processing.

RNA isolation, processing and qRT-PCR

Total RNA was extracted from freeze–dried mature-green fruit pericarps obtained from the second experimental set according to the modified ‘‘pine tree-method’’ (Guether et al. 2009) and spectrophotometrically quantified (Nanodrop Technologies, Inc.) checking the A260 nm/280 nm and A260/230 nm ratios to assess RNA purity. RNA was stored at -80°C for further processing. To obtain a pure DNA-free RNA extract, samples were subjected to DNAse treatment, using the turbo DNAfree TM kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. The treated RNA samples were then re-checked spectrophotometrically to determine the nucleic acid concentration, as previously described. The absence of DNA contamination was verified through amplification of a fragment of the tomato ubiquitin gene (Fiorilli et al. 2009) in a control reverse-transcription PCR using the OneStep RT-PCR Kit (Qiagen), followed by agarose gel electrophoresis. cDNA was synthesized from 750 ng of total RNA using the SuperscriptTM II Reverse Transcriptase Kit (Invitrogen) according to the manufacturer’s indications. cDNA was diluted 1:3 in sterile water for quantitative relative expression analysis (qRT-PCR). qRT-PCR was performed using a 48-well StepOneTM Real Time PCR system (Applied Biosystems). As a reference for normalization, a fragment of the tomato ubiquitin gene was amplified as described previously. For each condition, at least three biological replicates were obtained, each consisting of three technical replicates. Reactions were carried out in a final volume of 20 ll with 5 ll of water, 10 ll of iQ SYBR Green Supermix, 2 ll of forward and reverse primers (3 μM) and 1 μl of cDNA sample. PCR cycling program consisted of a holding stage of 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. A melting curve (55–95°C) with a heating rate of 0.5°C per 10 s and a continuous fluorescence measurement was recorded at the end of each run. Data were analyzed using the comparative threshold cycle method (DDCt) (Livak and Schmittgen 2001). Ct values were manually checked to eliminate outliers in technical replicates and the Ct of the target genes were normalized to the ubiquitin Ct values: DCt = Ct target- Ct ubiquitin. The mean DCt for each condition was considered and the fold change was finally calculated as 2-DDCt, where DDCt = DCt sample - DCt control. The log2(2-DDCt) was finally computed. All real-time PCR primers were designed using the Primer3 web software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), based on sequences downloaded from the Solgenomics database (http://solgenomics.net; Bombarely et al. 2011). The corresponding oligonucleotides (Table S2) were purchased from Sigma-Aldrich.

Phosphorus content measurement

Mature-green (MG) fruits collected from the second experimental set (also used for RNA extraction) were subjected to phosphorus content analysis. Pericarps of three fruits for each condition were freeze–dried overnight and milled to a powder using a mortar and pestle. A sample corresponding to 30 mg of fruit material was then extracted by incubating in 1 ml of 6 M nitric acid at 90°C for 1 h. Extracts were filtered and the filtrate was diluted 1:12 using distilled water. Phosphorus concentration in final extracts was measured using an Optima 7000 DV ICP-OES apparatus (PerkinElmer Instruments Ltd). Phosphorus standard solution (#P3869, Sigma) was used as reference.

Statistical analysis

Mycorrhizal parameters, biometrical data and phosphorus content values were analyzed using the non-parametric Kruskal–Wallis test (Kruskal 1952; Kruskal and Wallis 1952). The statistical significance was assessed at the 95 % level. Phenological trait parameters were checked for outliers using the 'outliers' R library (Komsta 2006) and then analyzed using a one-way ANOVA with Fisher's LSD post hoc test accepting significant differences with $P<0.05$. For qRT-PCR data, differences in values of cycles between different treatments were analyzed through a oneway ANOVA with Fisher's LSD post hoc test at $P<0.05$ level. All statistical analyses were performed in R environment (Bioconductor) using 'agricolae' (De Mendiburu 2012) and 'pgirmess' (Giraudoux 2015) packages, or using SYSTAT 10 statistical software.

Results

Screening of seven tomato ripening mutants for their responsiveness to AMF

To understand whether mutations that affect ripening in tomato also affect AM symbiosis, seven tomato ripening mutants were considered in a preliminary screening. We selected Gr, Nr, Nor and Rin, altered in ET metabolism, hp-1, altered in light signaling and pigment production and Tagl1-1 and Tagl1-11, silenced in two TOMATO AGAMOUS-LIKE (TAGL) MADS box genes (Vrebalov et al. 2009). Mutants were grown in parallel to their isogenic line ''Ailsa Craig'' under mycorrhizal colonization, inoculated with the AM fungus F. mosseae, and a control treatment. A first set of inoculated plants was grown under controlled conditions in a growth chamber and sampled at 60 DPI to assess AM root colonization level. A preliminary observation using a stereomicroscope showed a well expanded extra-radical mycelium (ERM) through all the root systems, with large amounts of newly developed spores. Quantification of AMF colonization (Table 1) showed that all the lines established successful colonization, as also confirmed by the presence of regularly-formed arbuscules. No statistically supported differences emerged between the wild type (WT) and mutant genotypes in any of the considered parameters (Table 1).

	F %	*M %*	*a %*	*A %*
Wt	50.41 ± 9.41^{a}	16.39 ± 6.64^{ab}	80.51 ± 7.10^{ab}	13.50 ± 6.40^{a}
Gr	44.0 ± 22.14^{a}	14.30 ± 7.95^{ab}	81.00 ± 40.65^{ab}	11.55 ± 6.38^{a}
hp-1	43.67 ± 23.82^{a}	16.63 ± 8.97^{ab}	80.78 ± 40.89^{ab}	13.64 ± 7.76^{a}
Nr	50.67 ± 29.72^{a}	11.87 ± 6.91^{b}	89.35 ± 44.76^{a}	10.69 ± 6.36^{a}
Nor	52.00 ± 30.09^{a}	14.98 ± 8.76^{ab}	84.11 ± 49.06^{ab}	12.50 ± 7.22^{a}
Rin	47.67 ± 25.90^{a}	19.78 ± 10.32^{a}	74.96 ± 39.69^{b}	15.21 ± 8.84^{a}
Tagl1-1	54.33 ± 28.02^{a}	16.24 ± 9.69^{ab}	87.48 ± 43.79^{ab}	14.32 ± 8.75^{a}
Tagl1-11	34.33 ± 20.53^{a}	9.51 ± 6.52^{b}	84.80 ± 43.65^{ab}	7.73 ± 4.98^{a}

Data are expressed as mean ± SD, ($n = 3$). Statistical data analysis was performed using the non-parametric Kruskal–Wallis test. Differences were accepted at $P < 0.05$ and are indicated with different letters

Table 1 Mycorrhization parameters estimated according to Trouvelot et al. (1986)

In a second experimental set, we studied the systemic responses of the seven ripening-altered lines growing in greenhouse under MYC and NM conditions across their complete life cycle. We monitored phenological and reproductive traits as the vegetative period length and the total number of flowers and fruits produced (Fig. 1). As expected, under AM colonization, the Ailsa Craig WT plants accelerated the floral transition, as previously observed in the Micro-Tom cv (Salvioli et al. 2012). For most of the mutants considered no statistically supported differences between MYC and NM were observed. However, Gr and Nor, which both display an altered ET perception, showed a significant increase in the vegetative period under MYC condition as opposed to WT. Flower and fruit production was not significantly affected by AM inoculation in WT (we only evidenced a positive trend in MYC plants). However, some of the ripening mutants revealed again a different pattern for these traits: hp-1 and Tagl1-1 produced significantly more flowers under MYC conditions and, conversely, Gr, Nr, and Nor (ET mutants) produced more flowers under NM conditions. Taken as a whole, this screening allowed us to conclude that all the seven mutations do not interfere with AM establishment in the roots, but some of them may have an effect on tomato phenology.

Gr and hp-1 mutant selection and characterization

On the basis of this first screening, two mutants were selected for a deeper analysis (i.e. Gr and hp-1) since they are involved in two diverse mechanisms regulating the ripening process. Gr is deficient in fruit ET perception and high-pigment-1 (hp-1), displays enhanced light signal perception throughout the plant, including the fruit. In addition, both of them have been well characterized (Liu et al. 2004; Barry and Giovannoni 2006) and are used also for commercial purposes. Both mutants showed similar AM colonization levels in roots (Fig. 2). The fruit phenotype of the two mutant lines confirmed previous characterizations (Mustilli et al. 1999; Barry and Giovannoni 2006). Hp-1 fruits monitored at the same ripening stage as WT showed enhanced fruit and leaf pigmentation, comparable to data reported in the tomato Genetics Resources Center database (http://tgrc.ucdavis.edu/Data/Acc/GenDetail.aspx?Gene=hp-1). Gr mutant fruits did not reach full maturity, turned yellowish and did not soften as WT, sporadically exhibiting red ripe patches at the center of the berry. Such a peculiar fruit phenotype mirrors a reduced perception of ET, with a probable indirect impact on carotenoid synthesis (Barry and Giovannoni 2006). The analysis showed that under control conditions (i.e. non-mycorrhizal), both hp-1 and Gr mutations led to an earlier flowering compared with WT. However, only the Gr mutant had significantly longer vegetative period in NM versus MYC conditions (opposite of WT). This also explains the major flower and fruit production in NM Gr (Fig. 1). When considering hp-1 mutant, even if no differences in the vegetative period length emerged between MYC and NM conditions, this genotype exhibited a statistically supported increase in the number of harvested fruits and produced most of the flowers under AMF colonization. These values were also significantly higher when hp-1 and WT were compared under MYC conditions. Thus, interestingly the two mutants displayed an opposite phenological behavior in respect to WT when comparing MYC and NM condition (Fig. 1). These results are consistent with the different role for ET and light signaling in mediating the AM effect in tomato fruit.

Gr and hp-1 show altered AM functioning at root and systemic level

To check the functionality of the colonization, the expression of the LePT4 gene, which is considered as functional marker of AM symbiosis, being mostly induced in arbusculated cells (Balestrini et al. 2007), was measured by qRT-PCR in roots (Fig. 3). Log2 fold-change values, considering the MYC WT as the steady-state, highlighted that LePT4 transcripts were significantly reduced in both mutants. Interestingly, both Gr and hp-1 genotypes exhibited similar values. To check the functionality of colonization at the systemic level, we measured fruit phosphorus levels in WT, Gr and hp-1 mature-green pericarps using ICP-OES (Fig. 4).

Fruit phosphorus level is enhanced systemically in fruits by symbiosis (Giovannetti et al. 2012) and it is considered as a valuable marker of the symbiosis at systemic level. Fruits from WT MYC plants showed an increase of about 30 % in the phosphorus content, similar to what has been previously reported (Hart et al. 2014). Interestingly, neither Gr nor hp-1 mutants showed any difference between MYC and control conditions, suggesting that one of the nutritional benefits caused by the symbiosis was lost in the mutated genotypes. However, the mutations in themselves slightly increased the phosphorus content, in that both genotypes showed intermediate phosphorus levels with respect to NM and MYC WT. These results confirmed both phenological data and LePT4 expression analyses, revealing that the systemic response to AMF was impaired in both mutants, also at the metabolic level. Taken as a whole, the experiments demonstrated that both mutants exhibit normal susceptibility to colonization but an altered functioning.

Expression pattern of ripening marker genes in Gr and hp-1 mutants

In the light of the above phenotypic, metabolic and gene expression results that revealed an unexpected impact of mycorrhization in some of the screened mutant collection we wondered whether genes which are considered as markers for the ripening process may reveal similar dynamics. With this aim, the expression of phytoene synthase (PSY1) a key gene in carotenoid biosynthesis activation, and of ETR4 and ETR6, two ET receptors (ETRs) known to be strongly expressed during fruit ripening, and previously associated with mycorrhizal effects (Torres de Los Santos et al. 2011), was investigated in mutant fruits under MYC and NM conditions versus the NM WT levels (Fig. 5). In WT, PSY gene was similarly expressed in MYC and NM conditions, even though a trend towards down-regulation under MYC treatment was observed in a previous RNA-seq experiment using the Moneymaker cultivar (Zouari et al. 2014). Lastly, no expression differences were detected between MYC and NM conditions in the mutant backgrounds. In Gr and hp-1 mutant fruits in NM conditions, PSY1 transcript levels were strongly reduced when compared with the WT (Fig. 5). This confirms previously reported data showing that PSY1 and PSY2 mRNA levels were lower in high-pigment mutants than the isogenic line at all the fruit ripening stages except fully-ripe in the hp-3 mutant (Galpaz et al. 2008). ETR4 and ETR6, as PSY1, were strongly regulated by the genotype rather than by mycorrhization. In the Gr background under control conditions, both ETR4 and ETR6 were down-regulated when compared to WT. This is in line with Gr characterization data, which shows a putative copper-binding activity for the GR protein (Barry and Giovannoni 2007); this trait may interfere with the activity of ET receptors including ETR4 and ETR6, since they require copper ions for their functioning (Rodrı´guez et al. 1999). Unlike the Gr, hp-1 genotype showed similar ETR4 transcript levels to WT under NM conditions (Fig. 5) while ETR6 was strongly up-regulated. When looking at gene expression upon AM colonization, no differential regulation of ETRs emerged, except for ETR6, which was interestingly slightly up-regulated by fungal inoculation in Gr mutant. These last data support the idea that the altered ET reception in Gr also impact ETR6 expression under AM colonization, resulting in the phenotype described before (Fig. 1, 2) In conclusion, while the ethylene (GR) and light/-carotenoid (HP1) mutations in NM conditions affect the expression of ripening marker genes related to ET response and carotenoid accumulation, mycorrhization does not substantially interfere with their expression in both WT and mutant backgrounds at the stages examined.

Discussion

Tomato responds well to AM symbiosis (Boldt et al. 2011) of which the systemic effects on fruit features are a potentially important aspect in the context of sustainable agriculture systems which have to also guarantee sufficient and healthy food (Ceballos et al. 2013). However, notwithstanding the many studies devoted to the impact of AMF on tomato biology, our knowledge on the potential relationships between

AM symbiosis and ripening is still limited. In this study, we investigated the interactions between AM symbiosis and the ripening process of tomato berries by comparing the response to AMF inoculation of seven ripening mutants to their isogenic Ailsa Craig WT line. Interestingly, all of them were susceptible to AM establishment, confirming results by Torres de Los Santos et al. (2011) and Fracetto et al. (2013) for Rin and Nr mutations, respectively. Using a combination of genetics, molecular and morphological tools we demonstrate that even if in the absence of a direct impact of mutations on the symbiosis establishment, a cross talk may exist between genetic changes impacting ET, light perception, and AM-induced systemic effects. This result suggests that whole plant and fruitspecific responses are influenced by AM interactions. Observation of mycorrhizal roots from the two mutants did not reveal any changes both in the morphology of the fungal structures and in the extent of the colonization. Such a result indicates that ET perception in the fruit and whole plant alterations in light signal transduction (both leading to strong effects on ripening phenotype and chemistry, respectively), do not affect mycorrhizal colonization levels or fungal structure morphology. These data originated by the seven mutant screening (Table 1) confirm the results of other studies on Nr and Rin mutations affecting ET metabolism (Torres de Los Santos et al. 2011; Fracetto et al. 2013). In these reports, only minor changes in AM quantification were detected and were limited to the first time points after the inoculation. Taken as whole, in an evolutionary perspective all these data suggest that genes involved specifically in the development of a red fleshy tomato fruit have little, if any, impact on the evolutionaryancient establishment of AM symbiosis. The results also suggest the absence of a cross talk between ripening and the symbiotic pathway (Oldroyd 2013) that regulates the early steps of mycorrhizal colonization. However, qRTPCR analysis of LePT4 which codes for a mycorrhizalinducible phosphate transporter (Balestrini et al. 2007; Gomez-Ariza et al. 2009) evidenced a significantly reduced expression level in two mutant lines (Gr and hp-1), suggesting that the mutations interfere with the functionality of the arbusculated cells, but not with the accommodation of the fungus. Indeed, the arbuscule morphology resulted to be regular, even if no specific morphometric analysis was performed (Volpe et al. 2015). Interestingly, the functionality of the root LePT4 transporter has a systemic effect, since the fruits from the WT mycorrhizal plants possessed 30 % more Pi than those from non-mycorrhizal plants. By contrast, no difference was detected in the fruits produced by the mycorrhizal mutated plants when compared to the control situation. While mycorrhiza is not impacted in its establishment by altered fruit ET response or whole plant light response, perturbing these signaling systems has an impact on plant phenology both under MYC and NM conditions. Previous investigations performed using Micro-Tom tomatoes colonized by F. mosseae revealed a positive correlation between AM, systemic effects and phenology (Salvioli et al. 2012). Ailsa Craig cv MYC WT confirmed this trend having a significantly reduced vegetative period. Since Moneymaker cv. did not show such a positive correlation (Zouari and Bonfante, unpublished results), we suggest that tomato responses to AMF may be cultivar-dependent. The responses of the studied mutants upon AM colonization were quite diverse: the GR mutation coupled to mycorrhization led to a delayed flowering and a dramatic reduction in the number of flowers and fruits in comparison with the NM mutant. Results for the hp-1 mutant did not reveal any differences since the shortening of the vegetative period depending on the mutation was also maintained upon AM colonization. In conclusion, AM colonization has a negative impact on the plant life cycle in the genetic Gr background. It seems that—in the absence of intact fruit ET perception—the cost paid for the fungal presence is not adequately balanced and the energy required for flowering and fruiting is acquired in longer times when compared with the NM or WT condition. These results suggest that ET and/or normal ripening progression in the fruit intersect with AM effects. By contrast, in the hp-1 mutant where there was enhanced pigment production and overall light sensitivity due to the mutation, the AM symbiosis acted as a positive regulator. The enhanced flower and fruit productivity under MYC conditions was probably favored by the synergy between the mutation itself (high light-sensibility in leaves, higher photosynthesis) and mycorrhization, which stimulates

photosynthesis both in leaves (Wright et al. 1998; Kaschuk et al. 2009; Schweiger and Muller 2015) and in fruits, at least as predicted on the basis of transcriptomic analysis (Zouari et al. 2014). ICP-OES analysis showed that while the fruits from the WT mycorrhizal plants possessed 30 % more phosphate than those from non-mycorrhizal plants, no differences were detected in the fruits produced by the mutant mycorrhizal plants. This implies that the mutations could have a broader impact on the whole fruit-transcriptome (Pan et al. 2014), activating may be other not AM-inducible transporters. In short, these observations indicate that normal ripening as mediated by ET, in addition to whole plant light responses, influence phosphate transport. They further suggest that perturbations likely elicit compensatory changes in response to ET-deficient fruit and aberrantly light-sensing plants.

Conclusion

Starting from an initial broad-range screening we picked out two mutants, affected in fruit-specific ethylene perception and whole plant light signaling and showed a link between fruit ripening and AM symbiosis. Even if both mutations had little effect on the establishment of mycorrhizal symbiosis at the root level, they led to an altered response in the systemic effects induced by the symbiosis, as summarized in Fig. 6. However, neither WT nor mutants showed a differential regulation of the tested ripening marker genes (PSY1, ETR4, ETR6) in their fruit under mycorrhizal conditions. We hypothesize that fruit ripening pathways linked to ET reception may overlap with other pathways used by the plants to activate systemic MYC responses in fruit. In this context, other hormones or miRNAs could be good candidates (Etemadi et al. 2014). Additionally, our data on hp-1 mutant support the hypothesis that the light signaling pathway may also act on plant systemic response to AMF. Ripening involved genes, such as GR or HP1, are clearly independent of the common symbiotic pathway (Oldroyd 2013) and are probably a more recent evolutive acquisition originated by the selection for fleshy fruit characteristics. Such an interpretation, if validated, may suggest that plants selected for fruit traits of human interest, as fruit weight (Lin et al. 2014), were also positively selected for their mycorrhizal systemic responsiveness. This could pave the way to investigate the role of AMF in tomato crop selection history.

Author contribution statement

PB, IZ, MC and AS conceived and designed the experiments. MC, IZ and MN conducted experiments. MC analyzed data. JJG and JV provided ripening mutants seed material and revised the final draft. PB and MC wrote the manuscript. All authors read and approved the manuscript.

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Fig. 1 Phenological parameters of Gr and hp-1 mutants in comparison to WT condition. a Vegetative period length (or days to flowering) expressed in DPI (days post inoculation). b Number of total flowers produced. c Number of total fruits produced. Data are expressed as mean ± SD (n = 5). Differences were tested using one-way ANOVA with Fisher's LSD post hoc test, P<0.05. Significant differences between conditions are marked with different letters. MYC mycorrhizal, NM non-mycorrhizal

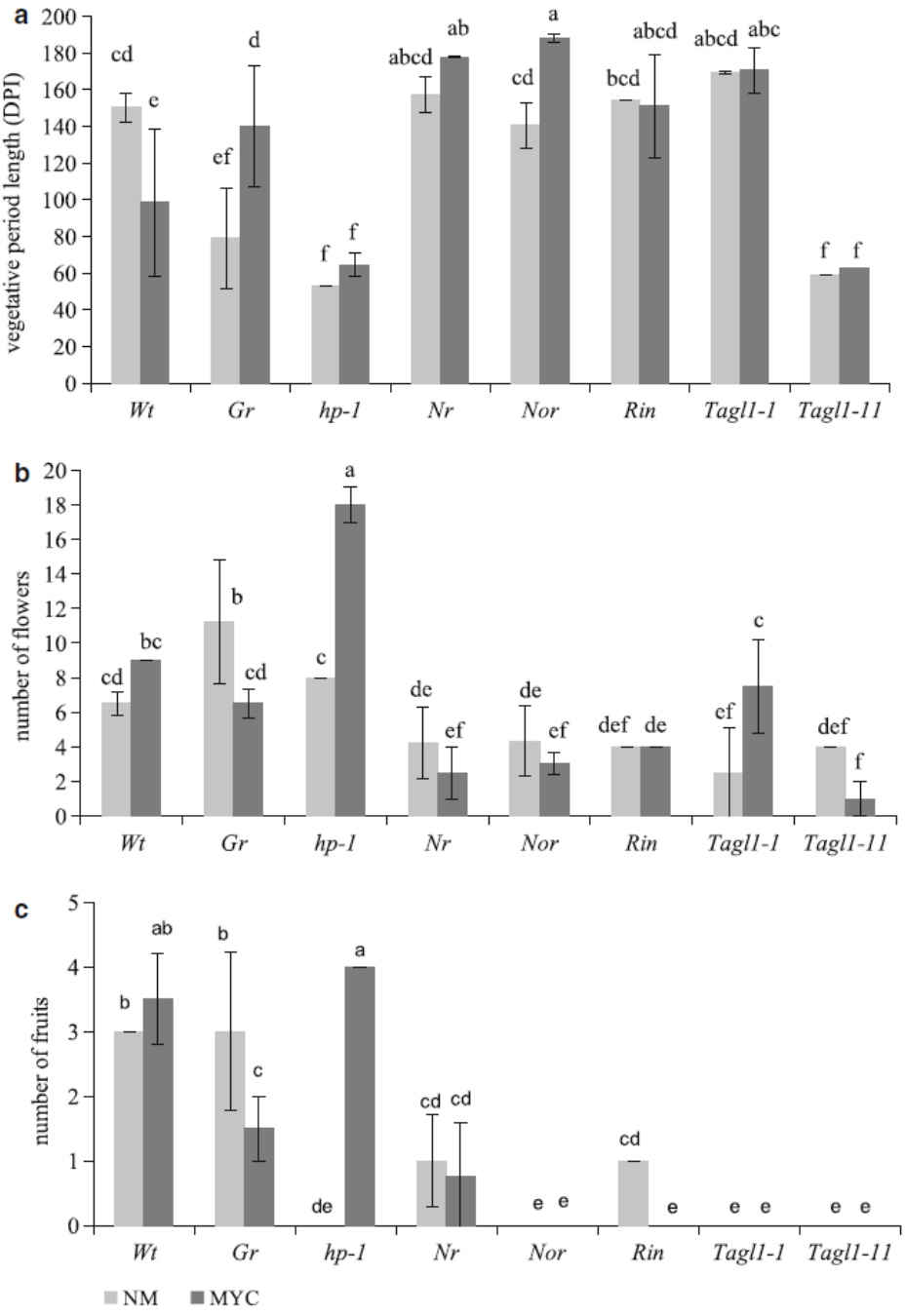

Fig. 2 Micro-photographs showing the level of intraradical fungal colonization in WT, Gr mutant and hp-1 mutant roots after cotton blue staining. Scale bar 1 mm

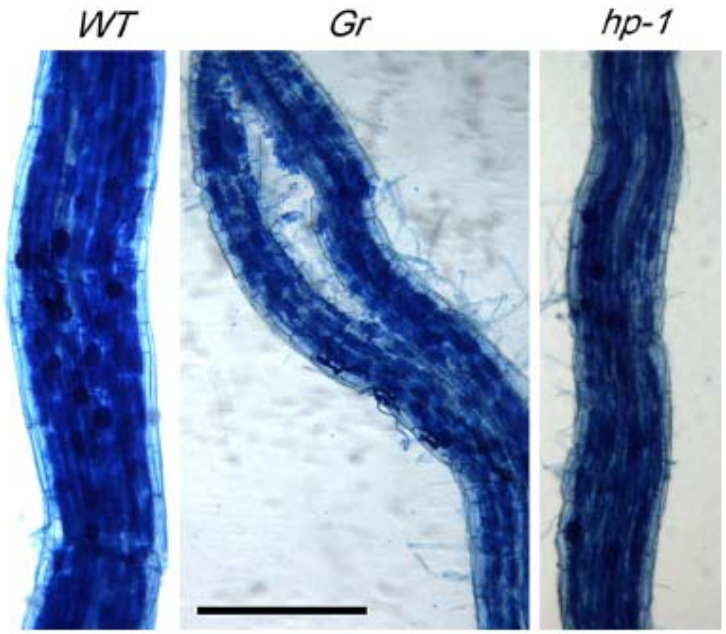

Fig. 3 qRT-PCR LePT4 expression in MYC WT and mutants root normalized to the MYC WT. Values are the mean log2 ratio ± SE of three biological replicates (with three qPCR technical replicates each). Statistically supported differences in DCt values were tested with a one-way ANOVA with Fisher's LSD post hoc test at $P<0.05$, n = 3 and are indicated with different letters

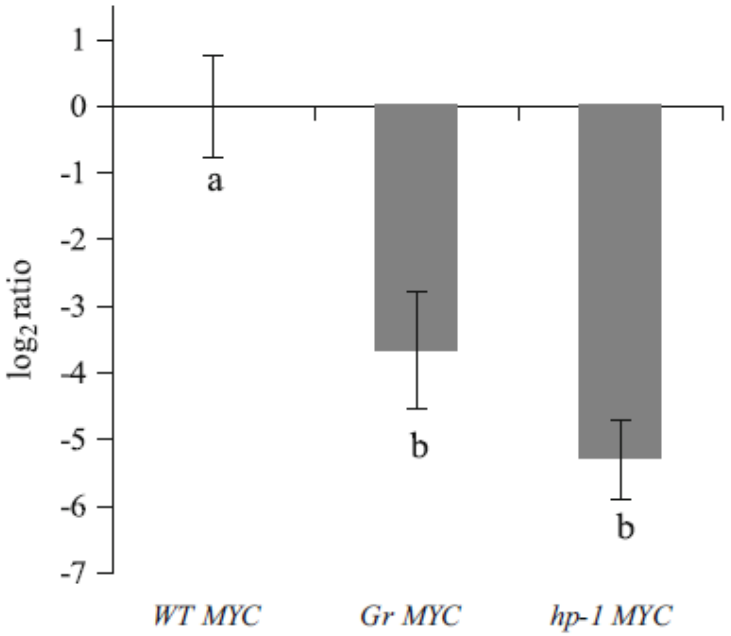

Fig. 4 Mature-green (MG) fruit phosphate concentration in WT, Gr and hp-1 genotype in NM and MYC conditions. Values are mean ± SD (n = 3). DW dry weight. Statistically supported differences are indicated with different letters (Kruskal–Wallis nonparametric test, P<0.05)

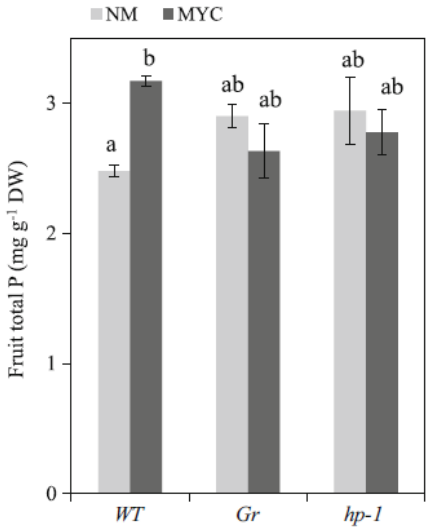

Fig. 5 qRT-PCR expression level of three marker-genes involved in tomato fruit ripening in mycorrhizal (MYC) and non-mycorrhizal (NM) mutants green-ripe berries. Values are the mean log2 ratio ± SE of three biological replicates (with three qPCR technical replicates each) normalized to the WT NM condition. Statistically supported differences in DCt values between MYC and NM condition were tested with a one-way ANOVA with Fisher's LSD post hoc test at P<0.05, and are indicated with different letters

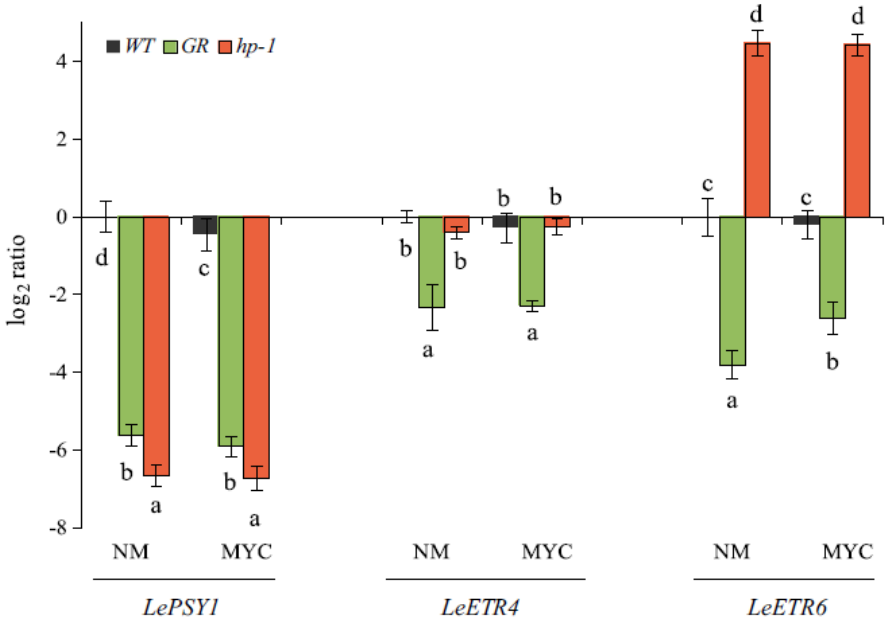

Fig. 6 The scheme illustrates how mutations in Gr (green fruits) and hp-1 (dark red fruits) genes do not impact the AM establishment and the fungal colonization. By contrast, the mutations have an impact not only on the fruit color at the fully ripe stage, but also on the systemic AM effects which are described in WT tomato at fruit level, also according to Zouari et al. (2014)